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Process for the fermentative preparation of L-amino acids using strains of the Enterobacteriaceae family

This invention relates to a process for the fermentative preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which the poxB gene is attenuated.

Prior art

L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition..(sic)

It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid

biosynthesis genes and investigating the effect on the production.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

Description of the invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which, in particular, already produce L-threonine and in which the nucleotide sequence which codes for the enzyme pyruvate oxidase (EC 1.2.2.2) (poxB gene) is attenuated.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

The process comprises carrying out the following steps:

a) fermentation of microorganisms of the Enterobacteriaceae family in which at least the poxB gene is attenuated,

b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and

c) isolation of the desired L-amino acid.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally

cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIgenetika MG442
Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000

Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate,

resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally a capacity for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feedback-resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feedback-resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feedback-resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after attenuation, in particular elimination, of the poxB gene, which codes for pyruvate oxidase (EC number 1.2.2.2).

It has furthermore been found that microorganisms of the Enterobacteriaceae family form lower concentrations of the undesirable by-product acetic acid after attenuation, in

particular elimination, of the poxB gene, which codes for pyruvate oxidase (EC number 1.2.2.2).

The nucleotide sequence of the poxB gene of Escherichia coli has been published by Grabau and Cronan (Nucleic Acids Research. 14 (13), 5449-5460 (1986)) and can also be found from the genome sequence of Escherichia coli published by Blattner et al. (Science 277, 1453 - 1462 (1997), under Accession Number AE000188. The nucleotide sequence of the poxB gene of Escherichia coli is shown in SEQ ID No. 1 and the amino acid sequence of the associated gene product is shown in SEQ ID No. 2.

The poxB genes described in the text references mentioned can be used according to the invention. Alleles of the poxB gene which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an attenuation, for example, expression of the poxB gene or the catalytic properties of the enzyme protein can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing, by genetic modification (mutation) of the signal structures of gene expression or also by the antisense-RNA technique. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information in this respect, inter alia, for example, in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999), Franch and Gerdes (Current Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or

that of Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95, 5511-5515 (1998), Wente and Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair in a gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

An example of a plasmid with the aid of which the poxB gene of Escherichia coli can be attenuated, in particular eliminated, by position-specific mutagenesis is the plasmid pMAK705ΔpoxB (figure 1). In addition to residues of polylinker sequences, it contains only a part of the 5' and

a part of the 3' region of the poxB gene. A 340 bp long section of the coding region is missing (deletion). The sequence of this DNA which can be employed for mutagenesis of the poxB gene is shown in SEQ ID No. 3.

The deletion mutation of the poxB gene can be incorporated into suitable strains by gene or allele replacement.

A conventional method is the method, described by Hamilton et al. (Journal of Bacteriology 174, 4617 - 4622 (1989)), of gene replacement with the aid of a conditionally replicating pSC101 derivative pMAK705. Other methods described in the prior art, such as, for example, those of Martinez-Morales et al. (Journal of Bacteriology 1999, 7143-7148 (1999)) or those of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)), can likewise be used.

After replacement has taken place, the strain in question contains the form of the ΔpoxB allele shown in SEQ ID No. 4, which is also provided by the invention.

It is also possible to transfer mutations in the poxB gene or mutations which affect expression of the poxB gene into various strains by conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the attenuation of the poxB gene.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene which codes for a corresponding enzyme or protein

with a high activity, and optionally combining these measures.

Thus, for example, one or more genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene which codes for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (DE 100 348 33.5),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765), and
- the thrE gene of Corynebacterium glutamicum which codes for threonine export (DE 100 264 94.8)

can be enhanced, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-amino acids, in particular threonine, in addition to the attenuation of the poxB gene, for one or more genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),

- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al., Archives in Microbiology 149, 36-42 (1987)),

- the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),

- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and

- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990))

to be attenuated, in particular eliminated or reduced in expression.

In addition to attenuation of the poxB gene it may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (feed process). A summary of known culture methods are [sic] described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und

periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51:. 1167-1174).

A pure culture of the Escherichia coli K-12 strain DH5α/pMAK705 was deposited as DSM 13720 on 12th September 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

A pure culture of the Escherichia coli K-12 strain MG442ΔpoxB was deposited as DSM 13762 on 2nd October 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The process according to the invention is used for the fermentative preparation of L-amino acids, such as e.g.

L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular cloning - A laboratory manual (1989) Cold Spring Harbour Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America USA (1989) 86: 2172-2175).

The incubation temperature for the preparation of strains and transformants is 37°C. Temperatures of 30°C and 44°C are used in the gene replacement method of Hamilton et. al.

Example 1

Construction of the deletion mutation of the poxB gene

Parts of the 5' and 3' region of the poxB gene are amplified from Escherichia coli K12 using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the poxB gene in E. coli K12 MG1655 (SEQ ID No. 1), the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

poxB'5'-1: 5' - CTGAACGGTCTTAGTGACAG - 3'

poxB'5'-2: 5' - AGGCCTGGAATAACGCAGCAGTTG - 3'

poxB'3'-1: 5' - CTGCGTGCATTGCTTCCATTG - 3'

poxB'3'-2: 5' - GCCAGTTCGATCACTTCATCAC - 3'

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturers instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany).

A DNA fragment approx. 500 base pairs (bp) in size from the 5' region of the poxB gene (called poxB1) and a DNA fragment approx. 750 bp in size from the 3' region of the poxB gene (called poxB2) can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Taq-DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are each ligated with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturers instructions and transformed into the E. coli strain TOP10F'.

Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml ampicillin are added. After isolation of the plasmid DNA, the vector pCR2.1TOPOpoxB1 is cleaved with the restriction enzymes Ecl136II and XbaI and, after separation in 0.8% agarose gel, the poxB1 fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After isolation of the plasmid DNA the vector pCR2.1TOPOpoxB2 is cleaved with the enzymes EcoRV and XbaI and ligated with the poxB1 fragment isolated. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin is added. After isolation of the plasmid DNA those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 3 is cloned are detected by control cleavage with the enzymes HindIII and XbaI. One of the plasmids is called pCR2.1TOPOΔpoxB.

Example 2

Construction of the replacement vector pMAK705ΔpoxB

The poxB allele described in example 1 is isolated from the vector pCR2.1TOPOΔpoxB after restriction with the enzymes HindIII and XbaI and separation in 0.8% agarose gel, and ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 174, 4617 - 4622), which has been digested with the enzymes HindIII and XbaI. The ligation

batch is transformed in DH5α and plasmid-carrying cells are selected on LB agar, to which 20 µg/ml chloramphenicol is added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with the enzymes HindIII and XbaI. The replacement vector formed, pMAK705ΔpoxB (= pMAK705deltapoxB), is shown in figure 1.

Example 3

Position-specific mutagenesis of the poxB gene in the E. coli strain MG442

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For replacement of the chromosomal poxB gene with the plasmid-coded deletion construct, MG442 is transformed with the plasmid pMAK705ΔpoxB, The gene replacement is carried out by the selection method described by Hamilton et al. (1989) Journal of Bacteriology 174, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers:

poxB'5'-1: 5' - CTGAACGGTCTTAGTGACAG - 3'

poxB'3'-2: 5' - GCCAGTTCGATCACTTCATCAC -3'

The strain obtained is called MG442ΔpoxB.

Example 4

Preparation of L-threonine with the strain MG442ΔpoxB

MG442ΔpoxB is multiplied on minimal medium with the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition:

2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this preculture are transinoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose) and the batch is incubated for 48 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442ΔpoxB	4.9	2.6

The following figure is attached:

- Figure 1: pMAK705ΔpoxB (= pMAK705deltapoxB)

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of the plasmid pSC101
- poxB1: part of the 5' region of the poxB gene
- poxB2: part of the 3' region of the poxB gene

The abbreviations for the restriction enzymes have the following meaning

- BamHI: restriction endonuclease from Bacillus amyloliquefaciens
- BglII: restriction endonuclease from Bacillus globigii
- ClaI: restriction endonuclease from Caryphanon latum
- Ecl136II restriction endonuclease from Enterobacter cloacae RFL136 (= Ecl136)
- EcoRI: restriction endonuclease from Escherichia coli
- EcoRV: restriction endonuclease from Escherichia coli

- HindIII: restriction endonuclease from Haemophilus influenzae
- KpnI: restriction endonuclease from Klebsiella pneumoniae
- PstI: restriction endonuclease from Providencia stuartii
- PvuI: restriction endonuclease from Proteus vulgaris
- SmaI: restriction endonuclease from Serratia marcescens
- XbaI: restriction endonuclease from Xanthomonas badrii
- XhoI: restriction endonuclease from Xanthomonas holcicola

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ggg gtg aaa cgc atc tgg gga gtc aca ggc gac tct ctg aac ggt ctt   96
Gly Val Lys Arg Ile Trp Gly Val Thr Gly Asp Ser Leu Asn Gly Leu
             20                  25                  30

agt gac agt ctt aat cgc atg ggc acc atc gag tgg atg tcc acc cgc   144
Ser Asp Ser Leu Asn Arg Met Gly Thr Ile Glu Trp Met Ser Thr Arg
         35                  40                  45

cac gaa gaa gtg gcg gcc ttt gcc gct ggc gct gaa gca caa ctt agc   192
His Glu Glu Val Ala Ala Phe Ala Ala Gly Ala Glu Ala Gln Leu Ser
     50                  55                  60

gga gaa ctg gcg gtc tgc gcc gga tcg tgc ggc ccc ggc aac ctg cac   240
Gly Glu Leu Ala Val Cys Ala Gly Ser Cys Gly Pro Gly Asn Leu His
 65                  70                  75                  80

tta atc aac ggc ctg ttc gat tgc cac cgc aat cac gtt ccg gta ctg   288
Leu Ile Asn Gly Leu Phe Asp Cys His Arg Asn His Val Pro Val Leu
                 85                  90                  95

gcg att gcc gct cat att ccc tcc agc gaa att ggc agc ggc tat ttc   336
Ala Ile Ala Ala His Ile Pro Ser Ser Glu Ile Gly Ser Gly Tyr Phe
            100                 105                 110

cag gaa acc cac cca caa gag cta ttc cgc gaa tgt agt cac tat tgc   384
Gln Glu Thr His Pro Gln Glu Leu Phe Arg Glu Cys Ser His Tyr Cys
        115                 120                 125

gag ctg gtt tcc agc ccg gag cag atc cca caa gta ctg gcg att gcc   432
Glu Leu Val Ser Ser Pro Glu Gln Ile Pro Gln Val Leu Ala Ile Ala
    130                 135                 140

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                165                 170                 175

tat cat gcg cca caa cca gtc gtg acg ccg gaa gaa gaa gag tta cgc   576
Tyr His Ala Pro Gln Pro Val Val Thr Pro Glu Glu Glu Glu Leu Arg
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Gly Ser Gly Cys Ala Gly Ala His Lys Glu Leu Val Glu Phe Ala Gly
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Lys Ile Lys Ala Pro Ile Val His Ala Leu Arg Gly Lys Glu His Val
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gaa tac gat aat ccg tat gat gtt gga atg acc ggg tta atc ggc ttc   768
Glu Tyr Asp Asn Pro Tyr Asp Val Gly Met Thr Gly Leu Ile Gly Phe
                245                 250                 255

tcg tca ggt ttc cat acc atg atg aac gcc gac acg tta gtg cta ctc   816
Ser Ser Gly Phe His Thr Met Met Asn Ala Asp Thr Leu Val Leu Leu
            260                 265                 270

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Gly Thr Gln Phe Pro Tyr Arg Ala Phe Tyr Pro Thr Asp Ala Lys Ile
        275                 280                 285

att cag att gat atc aac cca gcc agc atc ggc gct cac agc aag gtg   912
Ile Gln Ile Asp Ile Asn Pro Ala Ser Ile Gly Ala His Ser Lys Val
    290                 295                 300

gat atg gca ctg gtc ggc gat atc aag tcg act ctg cgt gca ttg ctt   960
Asp Met Ala Leu Val Gly Asp Ile Lys Ser Thr Leu Arg Ala Leu Leu
305                 310                 315                 320

cca ttg gtg gaa gaa aaa gcc gat cgc aag ttt ctg gat aaa gcg ctg  1008
Pro Leu Val Glu Glu Lys Ala Asp Arg Lys Phe Leu Asp Lys Ala Leu
                325                 330                 335

gaa gat tac cgc gac gcc cgc aaa ggg ctg gac gat tta gct aaa ccg  1056
Glu Asp Tyr Arg Asp Ala Arg Lys Gly Leu Asp Asp Leu Ala Lys Pro
            340                 345                 350

agc gag aaa gcc att cac ccg caa tat ctg gcg cag caa att agt cat  1104
Ser Glu Lys Ala Ile His Pro Gln Tyr Leu Ala Gln Gln Ile Ser His
        355                 360                 365

ttt gcc gcc gat gac gct att ttc acc tgt gac gtt ggt acg cca acg  1152
Phe Ala Ala Asp Asp Ala Ile Phe Thr Cys Asp Val Gly Thr Pro Thr
    370                 375                 380

gtg tgg gcg gca cgt tat cta aaa atg aac ggc aag cgt cgc ctg tta  1200
Val Trp Ala Ala Arg Tyr Leu Lys Met Asn Gly Lys Arg Arg Leu Leu
385                 390                 395                 400

ggt tcg ttt aac cac ggt tcg atg gct aac gcc atg ccg cag gcg ctg  1248
Gly Ser Phe Asn His Gly Ser Met Ala Asn Ala Met Pro Gln Ala Leu
                405                 410                 415
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ggt gcg cag gcg aca gag cca gaa cgt cag gtg gtc gcc atg tgc ggc   1296
Gly Ala Gln Ala Thr Glu Pro Glu Arg Gln Val Val Ala Met Cys Gly
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gat ggc ggt ttt agc atg ttg atg ggc gat ttc ctc tca gta gtg cag   1344
Asp Gly Gly Phe Ser Met Leu Met Gly Asp Phe Leu Ser Val Val Gln
        435                 440                 445

atg aaa ctg cca gtg aaa att gtc gtc ttt aac aac agc gtg ctg ggc   1392
Met Lys Leu Pro Val Lys Ile Val Val Phe Asn Asn Ser Val Leu Gly
    450                 455                 460

ttt gtg gcg atg gag atg aaa gct ggt ggc tat ttg act gac ggc acc   1440
Phe Val Ala Met Glu Met Lys Ala Gly Gly Tyr Leu Thr Asp Gly Thr
465                 470                 475                 480

gaa cta cac gac aca aac ttt gcc cgc att gcc gaa gcg tgc ggc att   1488
Glu Leu His Asp Thr Asn Phe Ala Arg Ile Ala Glu Ala Cys Gly Ile
                485                 490                 495

acg ggt atc cgt gta gaa aaa gcg tct gaa gtt gat gaa gcc ctg caa   1536
Thr Gly Ile Arg Val Glu Lys Ala Ser Glu Val Asp Glu Ala Leu Gln
            500                 505                 510

cgc gcc ttc tcc atc gac ggt ccg gtg ttg gtg gat gtg gtg gtc gcc   1584
Arg Ala Phe Ser Ile Asp Gly Pro Val Leu Val Asp Val Val Val Ala
        515                 520                 525

aaa gaa gag tta gcc att cca ccg cag atc aaa ctc gaa cag gcc aaa   1632
Lys Glu Glu Leu Ala Ile Pro Pro Gln Ile Lys Leu Glu Gln Ala Lys
    530                 535                 540

ggt ttc agc ctg tat atg ctg cgc gca atc atc agc gga cgc ggt gat   1680
Gly Phe Ser Leu Tyr Met Leu Arg Ala Ile Ile Ser Gly Arg Gly Asp
545                 550                 555                 560

gaa gtg atc gaa ctg gcg aaa aca aac tgg cta agg taa                1719
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Ser Asp Ser Leu Asn Arg Met Gly Thr Ile Glu Trp Met Ser Thr Arg
        35                  40                  45

His Glu Glu Val Ala Ala Phe Ala Ala Gly Ala Glu Ala Gln Leu Ser
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Gly Glu Leu Ala Val Cys Ala Gly Ser Cys Gly Pro Gly Asn Leu His
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Leu Ile Asn Gly Leu Phe Asp Cys His Arg Asn His Val Pro Val Leu
85 90 95

Ala Ile Ala Ala His Ile Pro Ser Ser Glu Ile Gly Ser Gly Tyr Phe
100 105 110

Gln Glu Thr His Pro Gln Glu Leu Phe Arg Glu Cys Ser His Tyr Cys
115 120 125

Glu Leu Val Ser Ser Pro Glu Gln Ile Pro Gln Val Leu Ala Ile Ala
130 135 140

Met Arg Lys Ala Val Leu Asn Arg Gly Val Ser Val Val Val Leu Pro
145 150 155 160

Gly Asp Val Ala Leu Lys Pro Ala Pro Glu Gly Ala Thr Met His Trp
165 170 175

Tyr His Ala Pro Gln Pro Val Val Thr Pro Glu Glu Glu Glu Leu Arg
180 185 190

Lys Leu Ala Gln Leu Leu Arg Tyr Ser Ser Asn Ile Ala Leu Met Cys
195 200 205

Gly Ser Gly Cys Ala Gly Ala His Lys Glu Leu Val Glu Phe Ala Gly
210 215 220

Lys Ile Lys Ala Pro Ile Val His Ala Leu Arg Gly Lys Glu His Val
225 230 235 240

Glu Tyr Asp Asn Pro Tyr Asp Val Gly Met Thr Gly Leu Ile Gly Phe
245 250 255

Ser Ser Gly Phe His Thr Met Met Asn Ala Asp Thr Leu Val Leu Leu
260 265 270

Gly Thr Gln Phe Pro Tyr Arg Ala Phe Tyr Pro Thr Asp Ala Lys Ile
275 280 285

Ile Gln Ile Asp Ile Asn Pro Ala Ser Ile Gly Ala His Ser Lys Val
290 295 300

Asp Met Ala Leu Val Gly Asp Ile Lys Ser Thr Leu Arg Ala Leu Leu
305 310 315 320

Pro Leu Val Glu Glu Lys Ala Asp Arg Lys Phe Leu Asp Lys Ala Leu
325 330 335

Glu Asp Tyr Arg Asp Ala Arg Lys Gly Leu Asp Asp Leu Ala Lys Pro
340 345 350

Ser Glu Lys Ala Ile His Pro Gln Tyr Leu Ala Gln Gln Ile Ser His
355 360 365

Phe Ala Ala Asp Asp Ala Ile Phe Thr Cys Asp Val Gly Thr Pro Thr
370 375 380

Val Trp Ala Ala Arg Tyr Leu Lys Met Asn Gly Lys Arg Arg Leu Leu
385 390 395 400

Gly Ser Phe Asn His Gly Ser Met Ala Asn Ala Met Pro Gln Ala Leu
405 410 415

Gly Ala Gln Ala Thr Glu Pro Glu Arg Gln Val Val Ala Met Cys Gly
420 425 430

Asp Gly Gly Phe Ser Met Leu Met Gly Asp Phe Leu Ser Val Val Gln
435 440 445

Met Lys Leu Pro Val Lys Ile Val Val Phe Asn Asn Ser Val Leu Gly
450 455 460

Phe Val Ala Met Glu Met Lys Ala Gly Gly Tyr Leu Thr Asp Gly Thr
465 470 475 480

Glu Leu His Asp Thr Asn Phe Ala Arg Ile Ala Glu Ala Cys Gly Ile
485 490 495

Thr Gly Ile Arg Val Glu Lys Ala Ser Glu Val Asp Glu Ala Leu Gln
500 505 510

Arg Ala Phe Ser Ile Asp Gly Pro Val Leu Val Asp Val Val Val Ala
515 520 525

Lys Glu Glu Leu Ala Ile Pro Pro Gln Ile Lys Leu Glu Gln Ala Lys
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Gly Phe Ser Leu Tyr Met Leu Arg Ala Ile Ile Ser Gly Arg Gly Asp
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Glu Val Ile Glu Leu Ala Lys Thr Asn Trp Leu Arg
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<222> (1)..(605)
<223> 5' region of the delta poxB allele

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<222> (606)..(674)
<223> Technical DNA/residues of the polylinker sequence

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<222> (675)..(1445)
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<222> (1446)..(1448)
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accatcgagt ggatgtccac ccgccacgaa gaagtggcgg cctttgccgc tggcgctgaa 180

gcacaactta gcggagaact ggcggtctgc gccggatcgt gcggccccgg caacctgcac 240
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taaggtaa 1448